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Voltage controlled nano-injection system for single-cell surgery

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Abstract

Manipulation and analysis of single cells is the next frontier in understanding processes that control the function and fate of cells. Herein we describe a single-cell injection platform based on nanopipettes. The system uses scanning microscopy techniques to detect cell surfaces, and voltage pulses to deliver molecules into individual cells. As a proof of concept, we injected adherent mammalian cells with fluorescent dyes.

Cell biology has traditionally been conducted by cell population studies as a way of gauging cell processes. While population dynamics provide a picture of the bulk behavior of a culture, greater attention is now warranted in elucidating the response at the single cell level[1], so as to differentiate among subpopulations of cells, which may have distinct responses from other cells within the same culture. This emerging field of "Single-Cell Biology" necessitates the development of tools capable of rapidly analyzing and manipulating individual cells in a semi-automatable fashion. Specifically, techniques are needed that can precisely introduce material to specific cells without affecting their viability. In particular, current methods to reprogram adult cells to induced plupripotent stem cells (iPSCs) are inefficient, presenting a significant barrier to their clinical application[2]. Despite intensive research, typically just 1 to 5% of cells reprogram[3]. The random nature by which each cell is modified complicates the systematic comparison of individual cells, particularly for experiments that seek to elucidate a stochastic response of individual cells to external stimuli. Methods for controlled cell injection have historically used glass micropipettes. Unfortunately, traditional micropipettes suffer from several drawbacks including large size relative to typical cells, low cell viability, lack of feedback and the requirement of a skilled operator[4, 5]. To reduce invasiveness, several nanofabricated structures have been used as direct methods of cell injection. A nanoneedle, fabricated on Atomic Force Microscopy (AFM) tips and coated with DNA was inserted into a single cell, and injection was accomplished by diffusion of DNA molecules from the nanoneedle[6]. Similarly, Cheng et al.

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developed an AFM based nanoinjector that uses a single carbon nanotube functionalized via disulfide bonds to deliver cargo into cells[7]. Alternative AFM based techniques have also been demonstrated but they are limited in terms of throughput and control of injection volumes[8, 9]. Laforge et al. developed an electrochemical attosyringe based on a glass nanopipette, that delivers liquid by applying a voltage across the liquid/liquid interface formed at a nanopipette filled with an organic solution and immersed into an aqueous one[10]. The resulting force is sufficiently strong to induce the flow of liquid into/out of the nanopipette. They have successfully used this effect to deliver attoliters of aqueous solution into mammalian cells in culture. Rodolfa et. al., have shown that double-barrel nanopipettes, fabricated from capillaries with theta-shaped cross sections, can be used for controlled deposition of biomolecules onto functionalized surfaces[11]. Further work has demonstrated deposition of material onto a surface in an inorganic solvent[12], and to cell membranes[13]. Unlike traditional micropipette injection techniques, the method relies not on pressure, but on applied voltage to expel liquid from the nanopipette. Our group pioneered the development of nanopipettes as a label-free sensing platform[14], which has been applied for the detection of cancer biomarkers[15], toxins[16], metal ions[17, 18], and to study precipitation at the nanoscale[19].

Here we present a fully-electrical system based on double-barrel nanopipettes capable of injecting a controlled amount of material into a cell. The system employs a Scanning Ion Conductance Microscope (SICM) that can position a nanopipette within a few hundred nanometers of the cell membrane[20]. The nanopipette is then precisely inserted in the cell cytoplasm and material is delivered by biasing one barrel with respect to the other (Fig. 1A). The amount of material delivered correlates with time and amplitude of the applied voltage (Fig. 1C). The implementation of double-barrel nanopipettes minimizes cell disturbance as no current is passed through the cell membrane, and physical membrane disruption is limited to well below 1 μm^2 (Fig. 1B). The double-barrel system also allows injection of multiple components into a single cell.

As a proof-of-principle, we chose human BJ fibroblast cells as targets for single-cell surgery. Thus, the challenge for the injection system was to locate cell surfaces and penetrate into the cytosol with minimal disruption. The cell injection system was designed to use a nanopipette both for feedback-controlled nanopositioning as well as delivery (Fig. S1). Aside from cell selection, all steps of the injection protocol are fully automated using the feedback control. First the nanopipette is lowered at steps of 3 nm to within ~100nm of the cell membrane where it enters feedback[21] (Fig. S5). The nanopipette is then quickly (100 μm/s) lowered 1 μm, penetrating the cell membrane. The low-voltage (≤1V) feedback system is disconnected from the nanopipette and a high-voltage (≥1V) source is connected by high-voltage relay for a predetermined amount of time. The nanopipette is then discharged through a small resistor, in order to minimize any transient bias, and then retracted at high-speed (100 μm/s). The entire protocol requires on average less than 1 minute per cell, and up to 10 cells can be injected in less than 5 minutes (Fig. S4).

We employed fluorescence microscopy to confirm the controlled delivery of carboxyfluorescein into human BJ fibroblasts. The precise control over injection time offers a means to regulate the amount of dye injected into cells. Fluorescence intensity increased in

a sigmoidal fashion with increasing duration of the applied voltage (Fig. 1C). Furthermore, three cells were injected with the same protocol (10V, 500ms) and the variation of fluorescence intensity among these cells was 17% (Fig. S6) which demonstrates the repeatability of nanopipette injections. When no electric field was applied, no fluorescence was measured demonstrating a negligible diffusion of dyes in the cell cytoplasm (data not shown).

Several groups developed nanoscale injection systems where the disruption to normal cellular biology is minimized throughout the configuration and protocol[22–26]. Nevertheless, none of their approaches allow simultaneous cell surface detection and control of the amount delivered. As shown in Fig. 1C, fluorescence arising from injected carboxyfluorescein at a penetration depth of 1μm is confined within the cell membrane post-injection. This result indicates that adherence of the cell membrane to the nanopipette tip, which would result in membrane shearing, does not occur — a result that is further confirmed by an absence of cell lifting during post-injection. Based on measurements of typical nanopipettes (tip radius approximately 50 nm, cone angle 8°, and penetration depth of 1 μm), the disruption to the cell membrane by the nanopipette should not exceed a pore of 500nm diameter, which is less than 0.1% of the surface area of a typical cell of 30μm in diameter. Notably, even at deeper penetration, cells do not lift from the culture surface and the same nanopipette tip can be used for subsequent injections, indicating that no damage or blockage of the tip occurs. We investigated long-term cell viability post-injection by injecting human BJ fibroblasts with carboxyfluorescein succinimidyl ester (CFSE), a fluorescent dye routinely used to track lymphocytes over successive cell divisions in rodent models[27]. The determination of optimal CFSE concentration, injection voltage and duration were important to achieving viability. We found that using voltages within the range 10–20V with durations of 0.2–2 seconds produce the best long-term viability with success ranging from 70–100%. Moreover, we observed cells injected to be fluorescent and with normal cell morphology 24 hours post injection. A further indication of cell viability includes observation of normal cell division 27 hours post-injection, which is in accordance with growth curves for this cell line. Following division, the daughter cells still show detectable fluorescence, which is absent in non injected cells, and appear to have normal cell morphology and migratory projections, typical of replicative fibroblasts (Fig. 2C). This result demonstrates the minimally invasive nature of the double-barreled nanopipette and the potential for this system to interrogate biologically relevant questions.

Using the double-barrel nanopipette for injection removes the requirement for an external reference electrode. Furthermore, it enables the independent injection of two separate materials, one from each barrel. We achieve the selective delivery of two distinct fluorescent dyes into human fibroblast cells, without cross-talk, from a single double-barrel nanopipette loaded with CFSE and sulforhodamine 101 (SR), each in separate barrels (Fig. 3A). Injection of both dyes into a single cell at varying ratios was also achieved, underscoring the possibility of being able to customize a specific cocktail of molecules for every cell (Fig. 3B).

The highly selective injection of material from only one channel at a time can be explained in terms of the electric field in the nanopipette.[11] In the two-channel setup, dye is only

ejected from the barrel positively biased. This is consistent with fluid delivery based on electroosmosis (EO), in which the nanopipette walls carry a negative charge with an electrical double layer of cations. The effect of EO is to drive the medium and its contents toward the nanopipette tip. Another force, electrophoresis (EP), acts on free ions in solution. These effects (EP and EO) can act together or against one another, determining at what bias a molecule will be ejected from the nanopipette[28–30]. The fact that no cross delivery was observed indicates that ejection in this case is dominated by electroosmosis (Fig 3, Fig S2). Neither CFSE nor SR dyes are highly charged; both carry a partial negative charge at neutral pH. For highly charged ions or particles with high zeta potential, different conditions may need to be used for multicomponent delivery. As shown in Figure 3B, the amount of material injected from each barrel can be controlled by the time of the applied voltage. A single cell injected with SR for 1000 ms and CFSE for 400 ms shows an overall yellow/orange color as a result of fluorescence from both the red and green channels. Another cell injected with the same nanopipette shows overall green fluorescence, a result of higher CFSE/SR ratio. This feature of the electrical injection device is particularly useful for both dose-response and multi-component injections, where many different conditions can be tested with a single nanopipette.

Because injection can be completed entirely inside the cell itself (without the need to retract and re-penetrate the plasma membrane), this methodology could, in theory, be extended to incorporate more chambers in the nanopipette, within the physical restraints of the system. Having multiple channels would increase the range of molecules that could be delivered to the cells using a single nanopipette.

Conclusions

We have demonstrated a technology for semi-automated, high viability cell injection. This double-barrel, voltage controlled nano-injection system addresses the need to deliver material into a cell controllably, reliably and without the need for highly specialized manual operations. The cell injection system, based on SICM technology to locate the cell surface and inject an arbitrary material into a single cell, demonstrates the robustness and speed required for efficient and effective research in Single-Cell Biology[31]. Cell viability was shown to be very high, and very little mechanical disruption to the cell was observed. The multi-component injection with a single nanopipette shows the potential of the technology to inject a customized cocktail of molecules into an individual cell, limited only by the number of barrels in a nanopipette. Such techniques are particularly useful for controlling cell fate.

The single-cell surgery technique demonstrated here can be used for manipulation of a variety of cell types. We are currently integrating this technology with microfluidics in order create a fully automated injection platform, applicable to non-adherent cells as well. Further experiments will be required to understand the factors that affect cell viability through healing of the cell membrane and response to voltage-driven injection. Additionally, the technique will benefit from a more complete understanding of the role of electrophoresis and electroosmosis on delivering particles of different size, charge, and rigidity. Ongoing studies in our group include the application of the nanosurgical platform to study the molecular mechanism involved in the reprogramming of human fibroblasts into induced pluripotent

stem cells (iPSCs) with synthetic modified mRNAs[3], and to study protein-RNA interactions at the single cell level[32].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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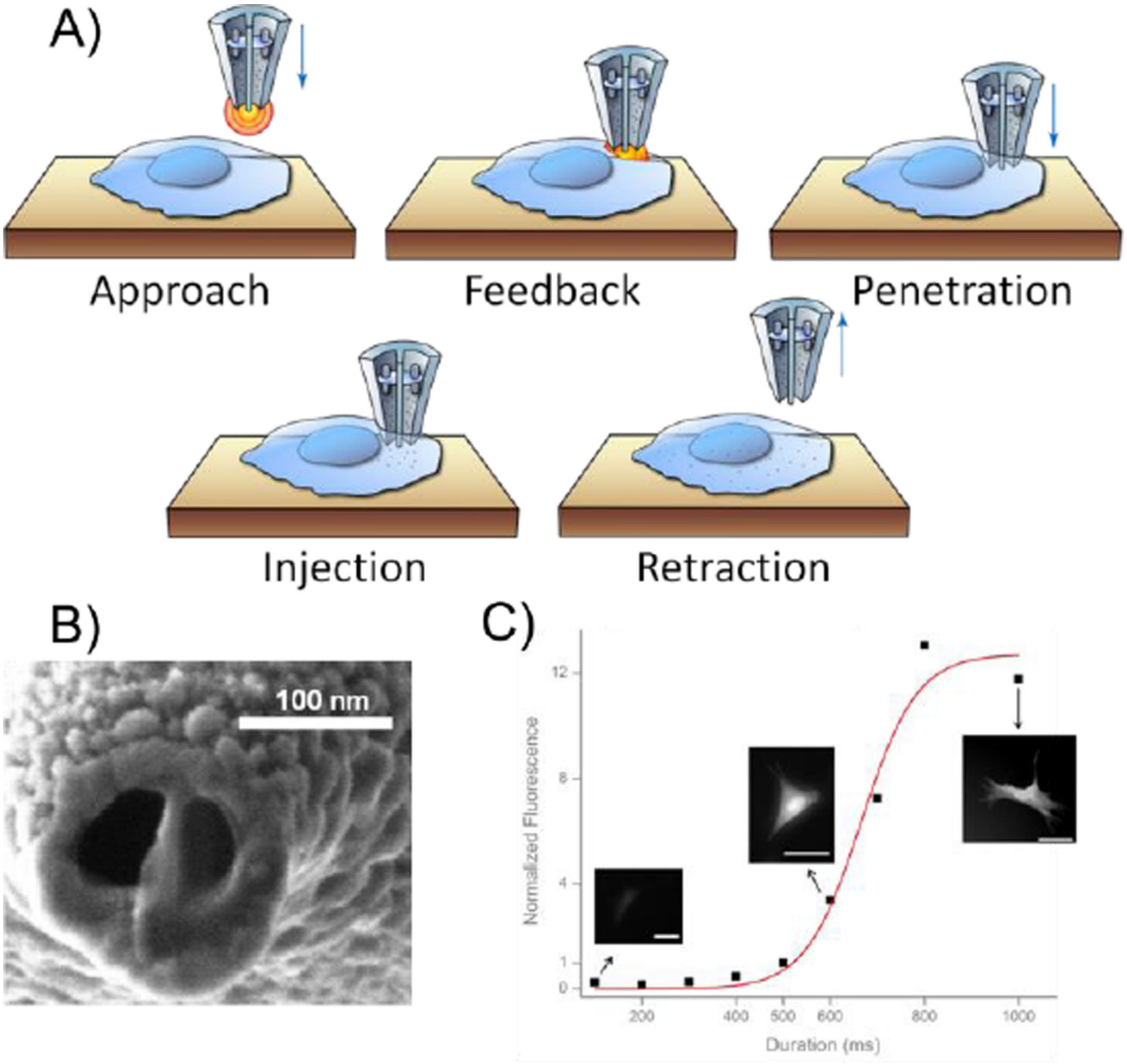

Fig. 1.

A) Illustration of cell surface detection, penetration and injection using a double-barrel nanopipette. B) SEM micrograph of gold sputtered double-barrel nanopipette. C) Injection of carboxyfluorescein into human BJ fibroblasts. The fluorescence intensity was normalized to the one measured at 500ms. Applied Voltage: 10V, scale bars 50 μm. Red curve is a sigmoidal fit to the experimental data point ($R^2 = 0.97$)

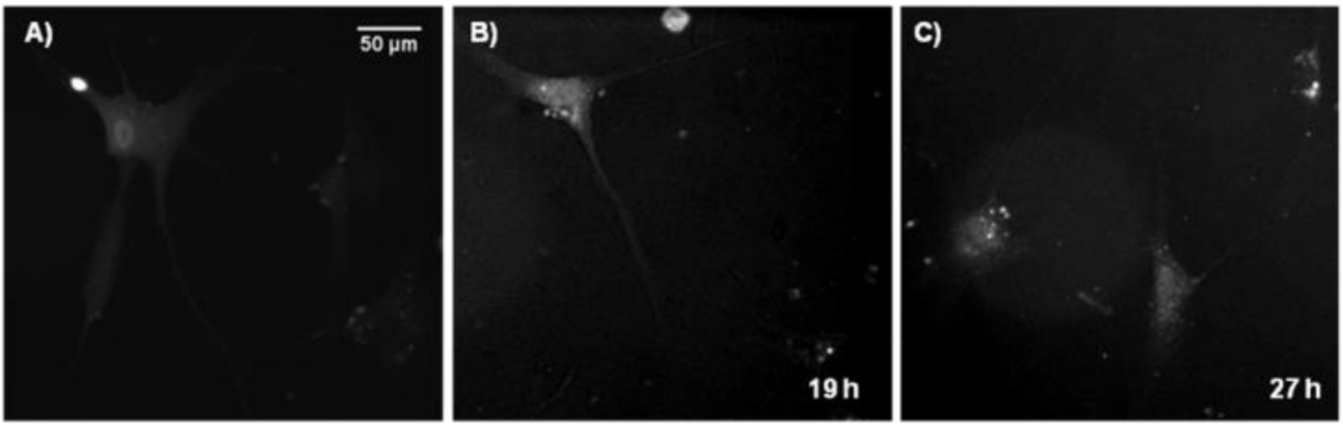

Fig. 2.

A) Human fibroblast cells 1 minute post injection of CFSE. Notice the bright nucleus of the injected cell with respect to other cells showing some amount of autofluorescence. This allows the subsequent localization of the same cell at later time points, despite changes in location and cell shape. B) The same cell visualized after 19 hours post injection. Fluorescence is diminished, but still significantly brighter than surrounding cells. C) Two daughter cells after a cell division event. The nucleus of both cells fluoresces relative to the cell body, as expected for two cells coming from a single injected cell. Image was taken 27 hours post injection.

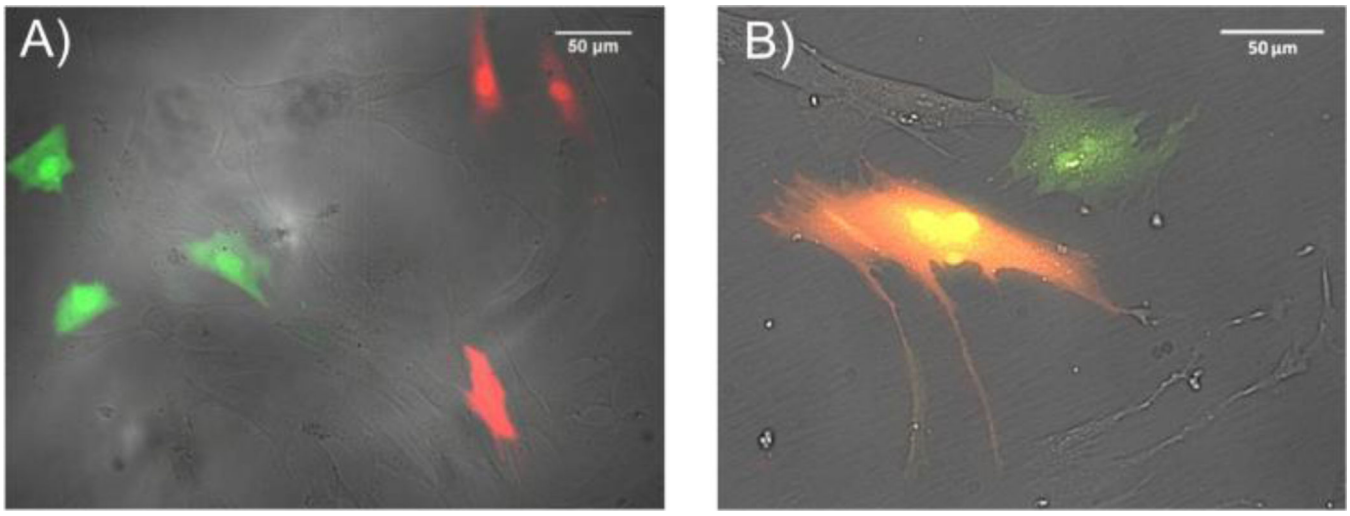

Fig. 3.

Multicomponent injection into human fibroblast cells. A) False color image of cells injected with a double-barrel nanopipette containing CFSE (green) and SR (red) in separate barrels (20V, 500 ms each). B) False color image of multicomponent injection with different dye ratios. A bias of 20 V was applied to each barrel for a fixed time to selectively inject one dye at a time. Left, yellow/orange color: 1000 ms applied to SR channel followed by 400 ms to CFSE channel. Right, green/yellow color: 400 ms applied to SR channel followed by 1000 ms to CFSE channel.